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THE INFLUENCE OF INNERVATION ON DIFFERENTIATING TONIC AND TWITCH MUSCLE FIBRES OF THE CHICKEN

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SUMMARY

1. The anterior (ALD) and posterior (PLD) latissimus dorsi muscles of adult chickens were denervated by section of their motor nerves. Four weeks later the contractile and membrane properties of these muscles were studied *in vitro* at room temperature.

2. Although the time course of the PLD muscle twitch was slightly prolonged, the qualitative difference in contractile characteristics of the slow ALD and the fast PLD muscles was maintained after denervation.

3. The difference in the passive membrane characteristics of the ALD and PLD muscle fibres was not lost after denervation, although the membrane resistance (R_m) and space constant (λ) of the denervated muscles fell. The membrane resistance, space (λ) and time (τ_m) constants of the ALD muscle remained significantly greater than for the PLD muscle fibres. The absolute values of τ_m in both muscles increased, implying that in the case of the ALD the membrane capacitance (C_m) was increased above normal after denervation. This is discussed in terms of the ultrastructural changes in this muscle after denervation.

4. The ALD muscle was cut into small pieces and replaced in the bed of the PLD muscle, which in turn was minced and placed into the bed of the ALD muscle. These muscles regenerated and became reinnervated by the PLD and ALD nerves respectively. They aligned themselves in the muscle bed and adopted the former shape of the muscle that they replaced. The passive cable properties of the regenerated ALD muscle fibres innervated by the PLD nerve resembled the control PLD fibres and the regenerated PLD fibres reinnervated by the ALD nerve resembled those of the control ALD. Regenerated ALD and PLD reinnervated by their own nerves had contractile and membrane properties similar to those of control muscles.

5. The results show that the fundamental differences, between slow and fast muscles once established, persist even when they are deprived of their innervation. The properties of developing muscle fibres however are determined by the motor nerves even in the adult animal.

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INTRODUCTION

The passive cable properties of the membranes of amphibian slow tonic and fast twitch muscles remain different after denervation or on reinnervation by an alien nerve. This difference is sufficiently great that individual fibres can be distinguished as tonic or twitch according to their membrane properties, even after denervation (Miledi & Stefani, 1970; Miledi, Stefani & Steinbach, 1971). It is possible that the membrane properties cannot be transformed by denervation and cross-innervation because they are predetermined properties of the muscles. This does not appear to be the case in avian muscle since the cable properties of slow and fast muscles of the chick are similar during early embryonic life and differentiate only at later stages of embryonic development and after hatching (Gordon, Purves & Vrbová, 1977). Although this differentiation takes place some time after innervation, it is not clear whether it is coincidental with the maturation of the muscle fibres, or whether it is induced by the particular nerve that contacts it.

The contractile and ultrastructural characteristics of the slow anterior latissimus dorsi (ALD) and the fast posterior latissimus dorsi (PLD) muscles are determined by the motor nerves during early development (Zelená & Jirmanová, 1973; Gordon & Vrbová, 1975*a*; Gordon, Perry, Srihari & Vrbová, 1977). We have studied whether the membrane properties also differentiate under the influence of innervation. Advantage was taken of the finding that when skeletal muscle is minced and replaced into the body of the animal, new myoblasts are formed that fuse into myotubes and form muscle fibres (see Carlson, 1973). In this study, the membrane properties of these newly regenerated muscle fibres were investigated to see whether they are determined by the motor nerve or by the parent muscle. The properties of ALD and PLD muscles were also studied after denervation in the adult animal to determine whether muscle properties, once established, are maintained in the absence of innervation, as they are in amphibian muscles.

In the adult chicken, the contractile and ultrastructural properties of the ALD and PLD muscles were not altered by cross-innervation (Hník, Jirmanová, Vyklický & Zelená, 1967). The results presented here show that the differences in the passive membrane characteristics and the contractile speed of slow and fast chick muscles are maintained after denervation. However, when ALD and PLD muscles were made to dedifferentiate and regenerate in the adult animal, the newly regenerated ALD muscle resembled the control PLD when it became innervated by the PLD nerve. Similarly, the cross-innervated regenerated PLD muscle has similar passive membrane and contractile properties to the control ALD muscle.

METHODS

Three week old white Leghorn chickens were anaesthetized with ether and the ALD muscle on one side and the PLD muscle on the other side were removed under aseptic conditions. The muscles were placed into separate sterile Petri dishes containing 0·3 ml. sterile saline and cut into small pieces (1–2 mm^2). The ALD pieces were then replaced either in their own bed or in place of the removed PLD and similarly for PLD pieces. Care was taken to avoid damage to the nerves to muscles that were not removed. The skin was closed and the animal left to recover. In another group of 3 week old chickens, the nerves to the ALD and PLD muscles were sectioned and a piece of nerve excised so as to prevent reinnervation.

Four weeks after the operation, the denervated and control muscles were excised, and 6–12 weeks

after the initial operation the regenerated and control muscles were removed and mounted in a bath containing oxygenated Krebs–Henseleit solution. One tendon was attached to a rigid bar and the other tendon to a strain gauge for tension recording. Contractions were elicited by direct maximal stimulation of the muscles using silver–silver chloride electrodes placed directly on to the muscle. Isometric tension was recorded and the length of the muscle was adjusted so as to produce the greatest twitch tension. The contractions were displayed either on an oscilloscope screen or on a Devices pen recorder.

After tension recordings were completed, the muscles were transferred into a different bath which was perfused with Krebs–Henseleit solution for recording of the passive electrical membrane properties of their fibres. The connective tissue was carefully removed from the surface of the muscle before recording. Intracellular electrical records were made by impaling fibres near their mid-point with KCl-filled glass micro-electrodes of resistance 20–60 MΩ. Inward current pulses (0·5–5 nA) passed through the recording electrode from an active bridge circuit evoked hyperpolarizing electrotonic potentials from which measurements of input resistance, R_{in}, and time constant, T_m (time to reach 83% of final value), were made. The current-voltage relation was linear for hyperpolarizations less than about 15 mV. Systematic errors in the measurement of R_{in} of the type described by Schanne, Kawata, Schäfer & Lavallée (1966) were avoided by the balancing procedure advocated by Engel, Barcilon & Eisenberg (1972).

The membrane resistance, R_m, space constant, λ, and membrane capacitance, were calculated and the results treated as described previously (Gordon *et al.* 1977). The mean values for the passive cable properties shown in the Figures and Tables are geometric means (cf. Gordon *et al.* 1977).

RESULTS

Denervated ALD and PLD muscles

Four weeks after nerve section the contractile properties of the denervated ALD and PLD muscles were compared to those of the contralateral control muscles. Fig. 1 shows records of the isometric contractions developed by control and denervated ALD and PLD muscles in response to direct electrical stimulation *in vitro* at room temperature. Although ALD muscles do not normally twitch in response to a single shock to the motor nerve (Hník *et al.* 1967), they develop twitch contractions in response to direct muscle stimulation, even *in vivo*. The time course of the twitch of control ALD was much longer than that of the PLD. The PLD contracted and relaxed about 3 times as rapidly as the slow ALD, as reported previously (Gordon & Vrbová, 1975*a*); the time to peak tension of the PLD in this study was $70{\cdot}4 \pm 3{\cdot}8$ msec ($n = 12$), as compared with $261 \pm 20{\cdot}4$ msec for the twelve ALD muscles (Table 1). The difference in the speed of contraction between the two muscles was also evident in tetanic contractions in response to stimulation at 40 Hz.

After denervation the difference between the two muscles persisted, although the time course of contraction of the muscles was altered (see Fig. 1 and Table 1). The time course of the twitch of PLD muscles was slightly prolonged after denervation, while there was no change in the tetanic contractions. The tetanic contractions of the ALD muscles were considerably longer after denervation. Thus, as a result of the much greater slowing effect of the denervation on ALD muscles the difference in the time course of tetanic contractions between the two muscles increased.

Passive membrane characteristics

Measurements of the time constant, τ_m and input resistance, R_{in}, were made in twenty-seven to fifty-three fibres from four denervated and four control ALD and PLD muscles using a single micropipette to pass cathodal current and to record voltage changes.

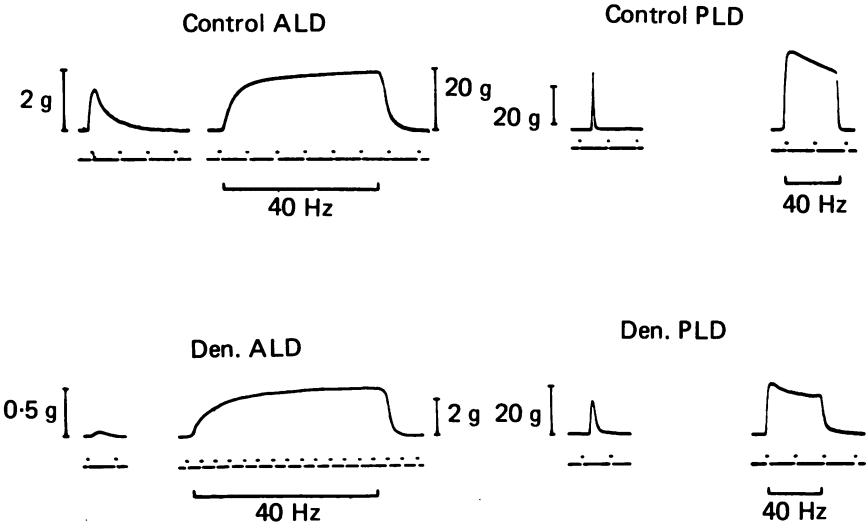

Fig. 1. Isometric tension developed by 4 week denervated ALD and PLD muscles and their contralateral control muscles in response to a single shock and repetitive stimulation at 40 Hz. Time markers occur every second.

TABLE 1. Speed of contraction of control and denervated muscles, the diameter and passive cable properties of fibres from these muscles. Mean values and S.E. are shown. The means of all parameters for ALD and PLD muscle fibres were significantly different in both control and denervated muscles, except of the diameter of fibres from control muscles.

	Control		Denervated	
	ALD	PLD	ALD	PLD
Twitch ttp (msec)*	261 ± 20	70·4 ± 3·8	280 ± 61	110·5 ± 18·1
Tet. ½ ttp (msec)	449 ± 45	62·5 ± 7·9	1102 ± 168	58·8 ± 11·4
Diam. (μm)	48·6 ± 8·1	46·2 ± 9·3	56·7 ± 10·9	22·6 ± 4·9
R_{in} (MΩ)	1·02 ± 0·10	0·41 ± 0·06	0·50 ± 0·04	1·29 ± 0·06
R_m (kΩ cm²)	6·73 ± 1·24	1·0 ± 0·27	2·47 ± 0·39	1·38 ± 0·17
τ_m (m)	42·5 ± 8·2	3·8 ± 0·5	55·9 ± 4·3	7·8 ± 1·0
λ (mm)	2·19 ± 0·21	0·80 ± 0·12	1·45 ± 0·12	0·67 ± 0·05

* ttp: time-to-peak tension.

In control ALD muscle fibres R_m was approximately 7 times greater than in PLD fibres (Table 1). Input resistance and R_m in ALD fibres fell after denervation, and since ALD fibres did not change in size (Table 1), the twofold decline in R_{in} was related directly to the fall in R_m: (R_{in} denervated/R_{in} control)^2 = R_m denervated/R_m control. PLD muscle fibres atrophied after denervation, as shown previously (Feng, Wu & Wang, 1965), and the increase in R_{in} in denervated PLD fibres was accounted for almost completely by the smaller diameter of the fibres. Thus values of R_m declined in the denervated ALD fibres and remained the same in denervated PLD (Fig. 1). Similar changes were seen for the space constant, λ, which fell to about 60 % of control value in denervated ALD muscle fibres, as predicted from the fall in R_{in}, and was

unchanged in denervated PLD fibres. Fig. 2 and Table 1 shows that while R_m and λ of ALD muscle fibres decrease after denervation ALD fibres remain significantly different from denervated PLD fibres, so that denervation reduced but did not abolish the difference between the membrane properties of tonic and twitch avian muscle fibres. The difference in τ_m between the muscles was however greater after denervation (Fig. 2*B*).

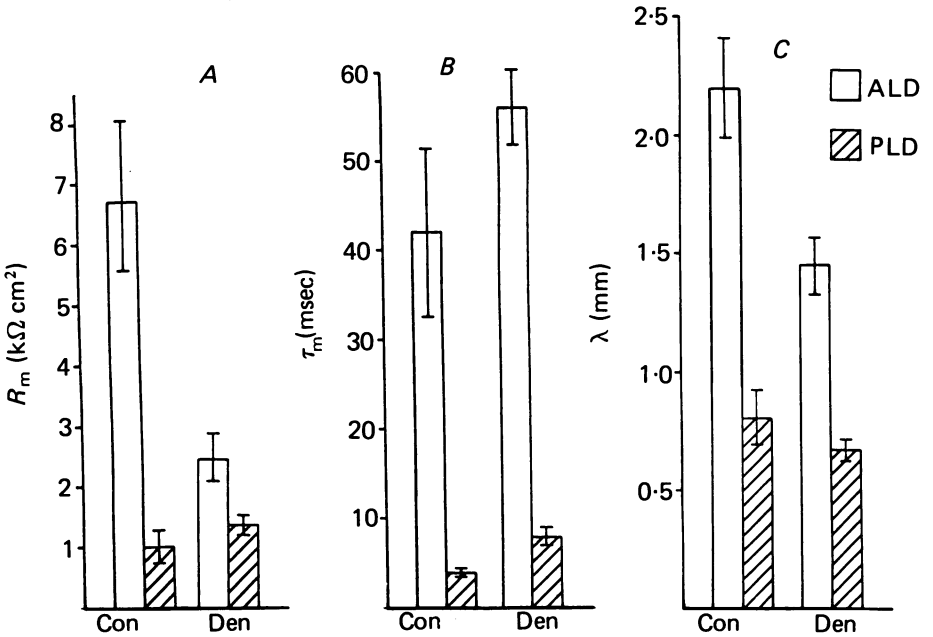

Fig. 2. Bar graphs showing the mean ± S.E. of (*A*) membrane resistance (R_m), (*B*) the time constant (τ_m) and (*C*) space constant (λ) of control and denervated ALD and PLD muscle fibres.

Contractile and membrane properties of regenerated muscle fibres

In six animals ALD muscle pieces were placed in the bed of a PLD muscle. The newly regenerated muscle fibres became innervated by PLD nerve, and their innervation pattern as well as their contractile properties resembled the original PLD muscle as shown previously (Gordon & Vrbová, 1975*a*, *b*). When in another group of six animals PLD was minced and replaced a normal ALD muscle, the regenerated muscle fibres became multiply innervated by ALD nerves and were slow contracting.

The input resistance and τ_m of 40–60 fibres from control and cross-reinnervated regenerated muscles were measured from electrotonic potential records, as shown in the examples in Fig. 3. From these values and measurements of muscle fibre diameters from frozen sections of the muscles, R_m was calculated. The differences between control ALD and PLD muscle fibres are shown in the bar graphs in Fig. 4. It is clear from these results that when ALD muscles regenerated and became reinnervated by PLD nerves, the membrane properties were not those of the original muscle, but had become determined by the PLD nerve: the membrane properties of cross-reinnervated ALD muscle were indistinguishable from the control PLD muscle fibres. Similarly,

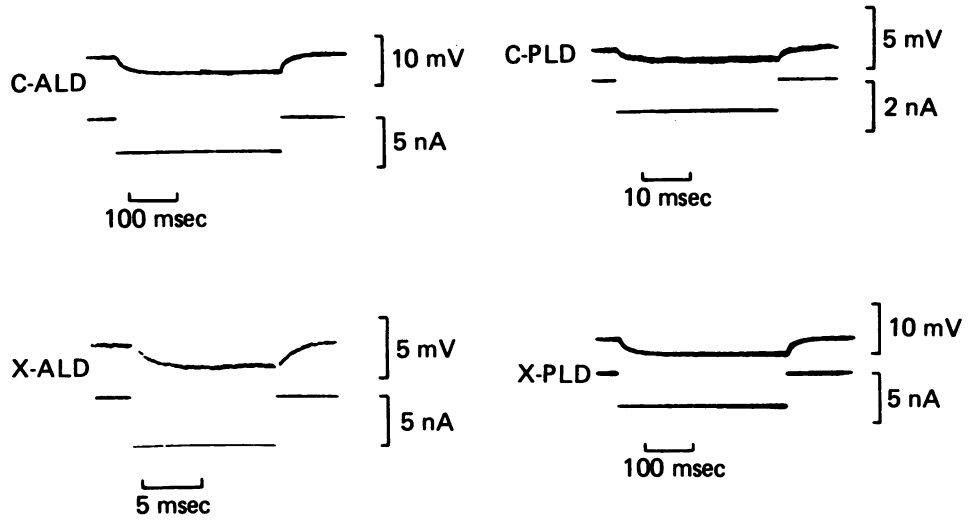

Fig. 3. Electrotonic potentials recorded in control and regenerated ALD and PLD muscle fibres in response to a negative pulse delivered by the same micropipette.

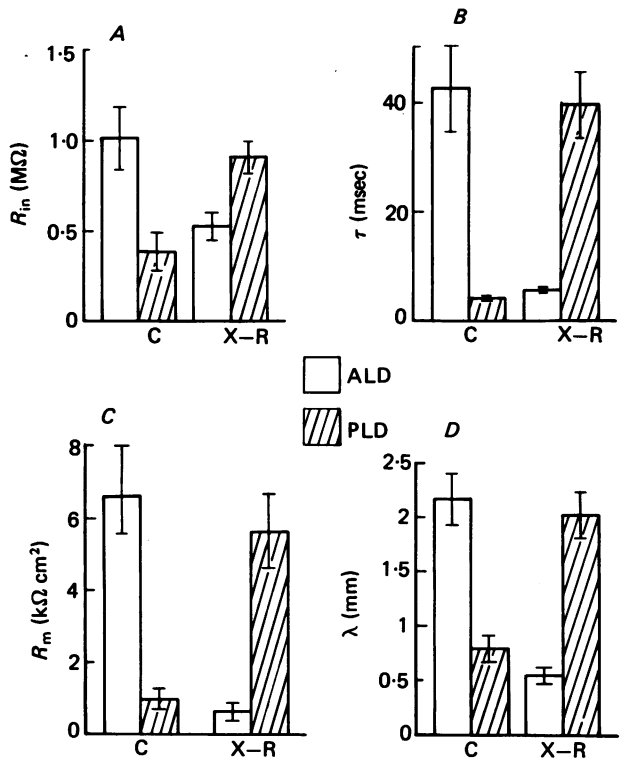

Fig. 4. Bar graphs of the mean ± S.E. of (*A*) the input resistance (R_{in}), (*B*) the time constant (τ_m), (*C*) membrane resistance (R_m), and (*D*) space constant (λ) of control and cross-innervated regenerated (X–R) ALD and PLD muscles.

the cross-reinnervated regenerated PLD muscle fibres acquired membrane properties identical to fibres from the control ALD muscle.

In another group of experiments either ALD or PLD muscles were removed, minced and replaced into their own original bed. Such self-reinnervated and regenerated ALD muscles were slow and PLD muscles fast contracting and the time course of their twitch and tetanic contraction was similar to that of the control (Gordon & Vrbová, 1975*a*). Results summarized in Table 2 show that the passive cable properties of the fibres from these muscles also resembled those of the control muscles, although there was considerable scatter in the computed values of R_m and λ. These were probably due to a wider scatter in fibre diameter.

These results taken together show that the regenerated muscle fibres always acquired the passive membrane properties of the muscle fibres that they replaced, and that the adult motor nerve determined the cable properties of these developing muscle fibres.

TABLE 2. Passive cable constants for self-reinnervated regenerated muscles are compared with those for control muscles. Geometric means were calculated as described in the methods and +S.E. is shown in brackets. The differences between cable constants for ALD and PLD were similar for experimental and control muscles

	Self rein ALD	Self rein PLD	Control ALD	Control PLD
R_{in} (MΩ)	0·72 (+0·08)	0·228 (+0·03)	1·0 (+0·10)	0·41 (+0·05)
τ_m (msec)	42·3 (+3·4)	4·35 (+0·94)	42 (+9·5)	3·8 (+0·45)
R_m (kΩ cm²)	3·46 (+1·31)	0·30 (+0·13)	6·7 (+1·33)	1·0 (+0·28)
λ (mm)	1·57 (+0·45)	0·45 (+0·15)	2·3 (+0·21)	0·8 (+0·12)

DISCUSSION

The passive cable properties of slow tonic fibres in ALD muscles are very different from those of the fast twitch fibres in PLD muscles, and ALD fibres are readily distinguishable by their longer time and space constants and their high membrane resistance, as well as differences in contractile speed (Ginsborg, 1960; Fedde, 1969; Hnik *et al.* 1967). The present results show that the fundamental differences in properties of the two muscles persist even when they are deprived of their innervation: the membrane resistance in ALD fibres remains greater than in PLD fibres, and the time and space constants are up to ten times greater in ALD than PLD fibres. Denervation affects the properties of the slow tonic muscle fibres of the ALD considerably more than those of the twitch fibres. Normally the difference in the time constants of fibres from ALD and PLD muscles reflects the differences in their membrane resistance. Following denervation, the time constant in ALD fibres increased while the membrane resistance fell to about one third of normal values. This implies that the membrane capacitance of denervated ALD fibres is also an order of magnitude larger than normal. These changes in passive membrane characteristics of ALD fibres are indicative of substantial alteration in their membranes. Hikida & Bock (1976) found that the amount of sarcoplasmic reticulum in slow tonic muscles increased after denervation. Although these membranes are not as well organized as

in the fast twitch muscles, the relative increase in membranes in denervated ALD muscles could account for the increased capacitance.

The membrane properties of the fast twitch fibres of the PLD do not change much after denervation. Although accuracy of measurements of passive cable properties with the single microelectrode method is low for cells with low input resistance and short time constants, our control values for input resistance and time constants differ less than twofold from those obtained in a small sample of fibres with a two micro-electrode method (Fedde, 1969). However, the technique that we used is not sufficiently sensitive to measure small changes in the membrane properties of PLD fibres after denervation accurately. No significant change in R_m and were detected, as shown in mammalian fast twitch muscles (Albuquerque & Thesleff, 1968; Albuquerque & McIsaac, 1970), although the time constant of the denervated PLD fibre in this study was significantly higher than in control innervated PLD muscle fibres, in agreement with the previous work in mammalian muscle. These findings on denervated avian tonic and twitch fibres are similar to previous reports on amphibians, where the difference between the properties of these two types of fibres persists after denervation, and where the membrane properties of tonic and twitch fibres were not transformed by alien innervation (Miledi & Stefani, 1970; Schmidt & Stefani, 1977).

This inability of amphibian tonic and twitch muscle fibres to change under the influence of innervation is in apparent contradiction with the results reported here, where the motor nerves determined the contractile and membrane properties of the regenerated ALD and PLD muscle fibres. The difference between the results obtained on cross-reinnervated amphibian and cross-reinnervated chicken muscles can most probably be explained by the fact that in this study developing muscle fibres were innervated by the alien motor nerve. Cross-reinnervation of adult ALD and PLD muscles does not induce a change of contractile or ultrastructural properties (Hník *et al.* 1967) and while there are no reports of the cable properties of cross-reinnervated ALD and PLD muscle fibres there is no reason to believe that they would be different from the amphibians, and change under the influence of an alien nerve. When however cross-reinnervation is performed on ALD and PLD muscles of newly hatched chicks their ultrastructural and contractile properties are determined by the motor nerve (Zelená & Jirmanová, 1973; Jirmanová, Hník & Zelená, 1971), suggesting that unlike mature muscle fibres, developing muscle fibres can be altered by the alien innervation. We have found in a previous study that the cable properties of embryonic ALD and PLD muscle fibres were indistinguishable and differentiated during the course of development some time after innervation was established; the difference between the two muscles increased after hatching at 21 days (Gordon *et al.* 1977). It was uncertain from that study whether the differentiation of the cable properties was correlated in time with the maturation of the muscle fibres or whether it was brought about by the motor nerve.

In the present experiments the muscle fibres that were reinnervated by their own or alien nerves were redeveloping in the adult birds. As during embryonic development, here too myoblasts fuse into myotubes to become muscle fibres. These newly formed muscle fibres become innervated by either ALD or PLD nerves (Ashurst & Vrbová, 1979), and their contractile and cable properties were determined by the respective motor nerves.

These findings taken together clearly show that the characteristic of developing tonic and twitch muscle fibres differentiate under the influence of their motor nerves. Once established they become a fixed property of the particular muscle fibre, which can be slightly modified, but not completely altered by interfering with its innervation. Thus at some stage during development, avian tonic and twitch fibres lose their potential to change.

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